Proton pump inhibitors affect the gut microbiome

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BACKGROUND AND AIMS
Proton pump inhibitors (PPIs) are among the top 10 most widely used drugs in the world. PPI use has been associated with an increased risk of enteric infections, most notably *Clostridium difficile*. The gut microbiome plays an important role in enteric infections, by resisting or promoting colonisation by pathogens. In this study, we investigated the influence of PPI use on the gut microbiome.

Methods The gut microbiome composition of 1815 individuals, spanning three cohorts, was assessed by tag sequencing of the 16S rRNA gene. The difference in microbiota composition in PPI users versus non-users was analysed separately in each cohort, followed by a meta-analysis.

Results 211 of the participants were using PPIs at the moment of stool sampling. PPI use is associated with a significant decrease in Shannon’s diversity and with changes in 20% of the bacterial taxa (false discovery rate <0.05). Multiple oral bacteria were over-represented in the faecal microbiome of PPI users, including the genus *Rothia* (p=9.8×10−38). In PPI users we observed a significant increase in bacteria: genera *Enterococcus*, *Streptococcus*, *Staphylococcus* and the potentially pathogenic species *Escherichia coli*.

Conclusions The differences between PPI users and non-users observed in this study are consistently associated with changes towards a less healthy gut microbiome. These differences are in line with known changes that predispose to *C. difficile* infections and can potentially explain the increased risk of enteric infections in PPI users. On a population level, the effects of PPI are more prominent than the effects of antibiotics or other commonly used drugs.

BACKGROUND AND AIMS
Proton pump inhibitors (PPIs) are among the top 10 most widely used drugs in the world. In 2013, 7% of the population of the Netherlands used omeprazole. In the same year, esomeprazole was the second largest drug in terms of revenue in the USA.1 2 PPIs are used to treat GORD and to prevent gastric and duodenal ulcers.3 4 Of the general population, 25% report having heartburn at least once a month, explaining the large demand for PPIs.4 Nevertheless, PPIs are frequently prescribed or taken for long periods without evidence-based indication.5 6 PPI use has been associated with increased risk of enteric infections.7 8 9 A meta-analysis of 23 studies, comprising almost 300 000 patients, showed a 65% increase in the incidence of *Clostridium difficile*-associated diarrhoea among patients who used PPI.9 In healthcare-related settings, PPI use also increases the risk of recurrent *C. difficile* infections.5 Another meta-analysis of 11 280 patients, from six studies evaluating *Salmonella*, *Campylobacter* and other enteric infections, also found an increased risk due to acid suppression, with a greater association with PPI than with H2-receptor antagonists.9 Recently, the Dutch National Institute for Public Health and the Environment noticed a marked increase in the occurrence of *campylobacteriosis* associated with increased PPI use in the Netherlands.7

The gut microbiome plays an important role in these enteric infections.10 11 Gut microbiota can
resist or promote the microbial colonisation of the gut by *C. difficile* and other enteric infections through several mechanisms that either directly inhibit bacterial growth or enhance the immune system. Moreover, substituting the gut microbiota of patients with *C. difficile*-associated diarrhoea with a healthy microbiome through faecal transplantation has been proven to

**Table 1** Characteristics of the three independent cohorts in this study

<table>
<thead>
<tr>
<th></th>
<th>Cohort 1: LifeLines-DEEP (general population)</th>
<th>Cohort 2: patients with IBD UMCG</th>
<th>Cohort 3: IBS case-control study MUMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPI users (n=99) Average (SD)*</td>
<td>Non-PPI users (n=1075) Average (SD)*</td>
<td>PPI users (n=52) Average (SD)*</td>
</tr>
<tr>
<td>Age</td>
<td>51.94 (13.59)</td>
<td>44.79 (13.58)</td>
<td>50.87 (14.49)</td>
</tr>
<tr>
<td>BMI</td>
<td>27.73 (5.10)</td>
<td>25.05 (4.03)</td>
<td>26.14 (5.53)</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>36.36</td>
<td>42.05</td>
<td>61.67</td>
</tr>
<tr>
<td>Reads per sample</td>
<td>48879 (43 001)</td>
<td>55 884 (40057)</td>
<td>51 081 (43 990)</td>
</tr>
<tr>
<td>Antibiotics (%)</td>
<td>2.02</td>
<td>1.02</td>
<td>31.67</td>
</tr>
<tr>
<td>IBD (%)</td>
<td>0.00</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>IBS (%)</td>
<td>34.34</td>
<td>25.77</td>
<td>0.00</td>
</tr>
<tr>
<td>Diarrhoea (%) (IBS-D and functional diarrhoea together)</td>
<td>7.07</td>
<td>4.47</td>
<td>–</td>
</tr>
<tr>
<td>Average bowel movements per day</td>
<td>1.36 (0.53)</td>
<td>1.38 (0.61)</td>
<td>–</td>
</tr>
<tr>
<td>Anti-TNF-a (%)</td>
<td>–</td>
<td>–</td>
<td>38.33</td>
</tr>
<tr>
<td>Mesoalazine (%)</td>
<td>–</td>
<td>–</td>
<td>26.67</td>
</tr>
<tr>
<td>Methotrexate (%)</td>
<td>–</td>
<td>–</td>
<td>16.67</td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>–</td>
<td>–</td>
<td>30.00</td>
</tr>
<tr>
<td>Thiopurines (%)</td>
<td>–</td>
<td>–</td>
<td>21.67</td>
</tr>
</tbody>
</table>

*Unless otherwise stated

IBD, Inflammatory Bowel Disease; IBS, Irritable Bowel Syndrome; SD, standard deviation; BMI, body mass index; MUMC, Maastricht University Medical Center; PPI, proton pump inhibitor; TNF-a, tumour necrosis factor; UMCG, University Medical Center Groningen.

**Figure 1** PPI-associated statistically significant differences in the gut microbiome. Meta-analysis of three independent cohorts comprising 1815 faecal samples, showing a cladogram (circular hierarchical tree) of 92 significantly increased or decreased bacterial taxa in the gut microbiome of PPI users compared with non-users (FDR<0.05). Each dot represents a bacterial taxon. The two innermost dots represent the highest level of taxonomy in our data: the kingdoms Archea and Bacteria (prokaryotes), followed outwards by the lower levels: phylum, class, order, family, genus and species. Red dots represent significantly increased taxa. Blue dots represent significantly decreased taxa. FDR, false discovery rate; PPI, proton pump inhibitor.
The increased incidence of enteric infections in PPI users and the importance of the gut microbiome composition in the development of these infections led us to investigate the influence of PPI use on the gut microbiome.

METHODS

Cohorts

We studied the effect of PPI use on the gut microbial composition in three independent cohorts from the Netherlands. These cohorts together comprise 1815 adult individuals, including healthy subjects and patients with GI diseases. Cohort 1 consists of 1174 individuals who participate in the general population study LifeLines-DEEP in the northern provinces of the Netherlands. Cohort 2 consists of 300 patients with IBD from the department of Gastroenterology and Hepatology University Medical Center Groningen (UMCG), the Netherlands. Cohort 3 consists of 189 patients with IBS and 152 matched controls from Maastricht University Medical Center (MUMC), the Netherlands.

Medication use

Current medication use at the time of stool collection of Cohort 1 participants was extracted from a standardised questionnaire. Two medical doctors reviewed all the medication for 1174 participants. PPI use was scored if participants used omeprazole, esomeprazole, pantoprazole, lansoprazole, dexlansoprazole or rabeprazole. To exclude other possible drug effects on the gut microbiome, medication use was scored in eight categories, allowing for later correction of parameters or exclusion of
certain participants. These categories were medication that: (1) changes bowel movement or stool frequency, (2) lowers triglyceride levels, (3) lowers cholesterol levels, (4) anti-diabetic medication (oral and insulin), (5) systemic anti-inflammatory medication (excluding NSAIDs), (6) topical anti-inflammatory medication, (7) systemic antibiotics, including antifungal and antimalarial medication, and (8) antidepressants including serotonin-specific reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), mirtazapine, and tricyclic antidepressants (TCAs). The definitions of these categories are described in the online supplementary appendix.

Analysis of drugs used in Cohort 2 was based on the IBD-specific electronic patient record in the UMCG. Current PPI use, as well as current IBD medication (mesalazines, thiopurines, methotrexate, steroids, tumour necrosis factor (TNF)-α inhibitors and other biologicals) were scored at the time of sampling by the gastroenterologist treating the patient with IBD. Current PPI consumption in the IBS case-control Cohort 3 was based on self-reported questionnaires. Pseudonymised data for all three cohorts was provided to the researchers.

Gut complaints and other clinical characteristics
Information on age, gender and body mass index (BMI) was available for all three cohorts. In Cohort 1, gut complaints were investigated using an extensive questionnaire that included defecation frequency and the Bristol Stool Scale. Possible IBS and functional diarrhoea or constipation were determined using self-reported ROME III criteria. The patients with IBD in Cohort 2 were diagnosed based on accepted radiological, endoscopic and histopathological evaluation. All the IBD cases included in our study fulfilled the clinical criteria for IBD. IBS in Cohort 3 was diagnosed by a gastroenterologist according to the ROME III criteria.

Stool and oral cavity mucus sample collection
A total of 1815 stool samples and 116 oral cavity mucus samples were collected. Cohorts 1 and 2 used identical protocols to collect the stool samples. Participants of cohort 1 and 2 were asked to collect one stool sample at home. Stool samples were frozen within 15 min after stool production in the participants’ home freezer and remained frozen until DNA isolation. A research nurse visited all participants to collect the stool samples shortly after production and they were transported and stored at −80°C. Participants of cohort 3 were asked to bring a stool sample to the research facility within 24 h after stool production. These samples were immediately frozen upon arrival at −80°C.

Oral cavity mucus samples were collected from 116 additional healthy volunteers using buccal swabs.

DNA isolation and analysis of microbiota composition
Microbial DNA from stool samples was isolated with the Qiagen AllPrep DNA/RNA Mini Kit (cat. # 80204). DNA isolation from oral cavity swabs was performed using the UltraClean microbial DNA isolation kit (cat.# 12224) from MoBio Laboratories (Carlsbad, California, USA). To determine the bacterial composition of the stool and oral cavity mucus samples, sequencing of the variable region V4 of the 16S rRNA gene was performed using Illumina MiSeq. DNA isolation is described in the Methods section of the online supplementary appendix.
Table 2  Taxa and microbiome aspects associated with PPI use and increased risk of *C. difficile* infection

<table>
<thead>
<tr>
<th>Taxa or microbiome aspect</th>
<th>Direction in PPI users that increases the risk of <em>C. difficile</em> infection</th>
<th>References of role on risk of <em>C. difficile</em> infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>α diversity</td>
<td>Reduced</td>
<td>Buffie et al.26 Nature, 2015</td>
</tr>
<tr>
<td>k._Bacteria</td>
<td>Decreased</td>
<td>Reeves et al.27 Gut Microbes, 2011</td>
</tr>
<tr>
<td>p._Firmicutes</td>
<td></td>
<td>Antharam et al.28 Journal of Clinical Microbiology, 2013</td>
</tr>
<tr>
<td>c._Clostridia</td>
<td></td>
<td>Schubert et al.29 Mbio, 2014.</td>
</tr>
<tr>
<td>o._Clostridales</td>
<td></td>
<td>Rea et al.30 Journal of Clinical Microbiology, 2011.</td>
</tr>
<tr>
<td>f._Ruminococcaceae</td>
<td></td>
<td>Buffie et al.31 Nature Reviews Immunology, 2013</td>
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<td>p._Actinobacteria</td>
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<td>c._Actinobacteria</td>
<td></td>
<td>Baines et al.34 Journal of Antimicrobial Chemotherapy, 2013</td>
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<tr>
<td>o._Bifidobacterales</td>
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<td></td>
</tr>
<tr>
<td>f._Bifidobacteriaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g._Bifidobacterium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k._Bacteria</td>
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<td>Antharam et al.28 The Journal of Clinical Microbiology, 2013</td>
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<tr>
<td>c._Bacilli</td>
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<td></td>
</tr>
<tr>
<td>o._Lactobacillales</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f._Lactobacillaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g._Lactobacillus</td>
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<td></td>
</tr>
<tr>
<td>s._debrueckii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s._plantarum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s._reuteri</td>
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<td>p._Proteobacteria</td>
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</tr>
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</tr>
<tr>
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<td></td>
<td>Peterfreund et al.35 PLOS ONE, 2012</td>
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<td>f._Enterobacteriaceae</td>
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<td></td>
</tr>
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<td>g._Escherichia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>s._coli</td>
<td></td>
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</tr>
</tbody>
</table>

**Taxonomy determination**

Bacterial taxonomy was determined by clustering the sequence reads with UCLUST (V1.2.2q) with a distance threshold of 97%, using Greengenes (V13.8) as the taxonomy reference database. Sequencing and the determination of taxonomy are described in the Methods section of the online supplementary appendix.

**Statistical analysis**

In each cohort, differentially abundant taxa in the gut microbiome between PPI users and non-PPI users were analysed using the multivariate statistical framework MaAsLin.17 MaAsLin performs boosted, additive, general linear models between metadata and microbial abundance data. After running the association studies in the individual cohorts, we performed a meta-analysis of the three cohorts, using the weighted Z-score method. The Cochran’s Q test was used to check for heterogeneity. The significance cut-off for the Cochran’s Q test was determined by Bonferroni correction for the 92 significant results: p<5.43×10⁻⁴. Differences in richness (the number of species within a sample), principal coordinate analyses (PCoA) and Shannon diversity analysis were determined using the QIIME microbiome analysis software.18 The Wilcoxon test and Spearman’s correlations were used to identify differences in Shannon’s diversity and relations between the PCoA scores of PPI users and non-PPI users, while the χ² test, Fisher’s exact test, Spearman’s correlation and Wilcoxon-Mann-Whitney test (WMW test) were used to determine differences in age, gender, BMI, antibiotics use, and gut complaints between PPI users and non-users. In all the microbiome analyses, multiple test corrections were based on the false discovery rate (FDR). An FDR value of 0.05 was used as a statistically significant cut-off.

In addition to the PPI effect, we also tested the influence of other commonly used drugs in Cohort 1. Using MaAsLin with similar settings to those described above, we tested the microbial
changes associated with the use of other drugs, with and without correction for PPI, and the changes when including these common drugs as a correcting factor in the PPI versus non-PPI analysis.

Significant results were graphically represented in cladograms using GraPhLAn. More details on the statistical analysis can be found in the Methods section (see online supplementary appendix).

**Correction for factors influencing the gut microbiota**

Differentially abundant taxa were corrected for several parameters, which were identified by statistical analysis of cohort phenotypes or univariate MaAsLin runs and subsequently added as cofactors to the additive linear model. Analyses in the general population Cohort 1 were corrected for age, gender, BMI, antibiotics use, sequence read depth and ROME III diagnosis (IBS-Constipation (IBS-C), IBS-Diarrhoea (IBS-D), IBS-Mixed (IBS-M), IBS-Undetermined (IBS-U), functional bloating, functional constipation, functional diarrhoea or none). The analysis of patients with IBD in Cohort 2 was corrected for age, gender, BMI, antibiotics use, sequence read depth, diagnosis (Crohn’s disease or UC) combined with disease location (colon, ileum or both) and IBD medication (use of mesalazines, steroids, thiopurines, methotrexate or anti-TNF antibodies). The analysis of the IBS case-control Cohort 3 was corrected for age, gender, BMI, sequence read depth and IBS status according to the ROME III criteria. In the meta-analysis, all microbiome data were corrected for age, gender, BMI, antibiotics use and sequence read depth.

**RESULTS**

**PPI use is associated with older age and higher BMI**

PPI were used by 211 (11.6%) of the 1815 participants: 8.4% of the general population (Cohort 1), 20.0% of the patients with IBD (Cohort 2) and 15.2% of the participants of case-control Cohort 3. Women use PPI more often than men: 9.2% versus 7.4%, albeit this was not statistically significant (p=0.61, χ² test). PPI users were generally older: 51.6 (SD 13.4) years of age versus 44.4 (SD 14.7) years of age (p=2.50×10⁻¹¹, WMW test) and have a higher BMI of 26.9 (SD 5.0) versus 24.9 (SD 4.2) for non-users (p=1.89×10⁻⁸, WMW test). Antibiotics were concomitantly used by 2% of the 99 PPI users of Cohort 1 and 33% of the 60 PPI users of Cohort 2. There was no overlap between PPI users and antibiotics users in Cohort 3. Based on our data, we included age, gender, BMI and antibiotics as cofactors in the microbiome analyses. Table 1 provides an overview of the characteristics per cohort and the use of PPI.

**Composition of the gut microbiota**

The predominant phylum in each cohort was Firmicutes with abundances of 76.7%, 73.8% and 77.4% in Cohorts 1, 2 and 3, respectively. Information on the composition of the gut microbiome for all three cohorts and on all taxonomic levels is provided in online supplementary figures S1, S2 and table S1. Independent of PPI use, the overall high-level bacterial composition of the gut was homogeneous in all three cohorts (by phylum, class and order level, Spearman’s correlations: r>0.94; p<1.6×10⁻¹⁵).

Reduced diversity of the gut microbiome associated with PPI use

In all three cohorts we identified a lower species richness and lower Shannon diversity, although not significant (Cohort 1, p=0.85; Cohort 2, p=0.16; Cohort 3, p=0.53), however in combined analysis of all three data sets we identified, moderate but significant decrease in gut α diversity of PPI users was observed in the meta-analysis of all 1815 gut microbiome samples: Shannon index (p=0.01) and species richness (p=0.02) (see online supplementary figures S3 and S4).

**Meta-analysis: differences in gut microbiome associated with PPI use**

The meta-analysis across all three cohorts showed statistically significant alterations in 92 of the 460 bacterial taxa abundance (FDR<0.05). These changes are depicted in a cladogram in figure 1 and in a heatmap in figure 2, and in online supplementary figure S5. Details of each taxon, including the individual direction, coefficient, p value and FDR for each cohort, as well as the meta-analysis, are provided in online supplementary tables S2 and S3. Cochran’s Q test was used to check for heterogeneity. None of the 92 reported associations were significantly heterogeneous at the Bonferroni corrected p value cut-off (p<5.43×10⁻⁴) (see online supplementary table S2).

The overall difference of the gut microbiome associated with PPI use was also observed in the PCoA of all the data sets together (figure 3 and see online supplementary figure S6). The same PCoA with separate colours for each cohort has been added in online supplementary figure S7. Notably, we observed statistically significant differences between PPI users and non-users in two principal coordinates (PCoA1: p=1.39×10⁻²⁰, PCoA3: p=0.0004, Wilcoxon test).

**Similar changes in three independent cohorts were associated with PPI use**

The order Actinomycetales, families Streptococcaceae, Micrococcaceae, genus Rothia and species Lactobacillus salivarius were increased in participants using PPI in each cohort. None of the individual cohorts contained any significantly decreased taxa (FDR<0.05). In the general population (Cohort 1), 41 of the 829 bacterial taxa were significantly increased, including the class Gammaproteobacteria, the family Enteroccaceae and the genera Streptococcus, Veillonella and Enterococcus (FDR<0.05) (see online supplementary table S4). In patients with IBD (Cohort 2), PPI use was associated with an increase of 12 of the 667 bacterial taxa, including the family Lactobacillaceae as well as the genera Streptococcus and Lactobacillus (FDR<0.05) (see online supplementary table S5). In IBS case-control Cohort 3, 18 of the 624 taxa were significantly increased, including the order Lactobacillales (FDR<0.05) (see online supplementary table S6).

**Oral cavity bacteria are more abundant in the gut microbiome of PPI users**

We hypothesised that the changes in the gut microbiome associated with PPI use are caused by reduced acidity of the stomach and the subsequent survival of more bacteria that are ingested with food and oral mucus. Indeed, some of the statistically significantly increased bacteria in PPI users (eg, Rothia dentocariosa, Rothia mucilaginosa, the genera Scardovia and Actinomyces and the family Micrococcaceae) are typically found in the oral microbiome. By analysing 116 oral microbiome samples from participants in Cohort 1, we could compare the overall composition of bacteria in the oral microbiome with the composition of the gut microbiome. We observed a statistically significant shift in Principal Coordinate 1 in the gut microbiome samples of the PPI users towards the oral samples, compared with non-PPI users (p=1.39×10⁻²⁰, Wilcoxon test) (figure 3). In online supplementary figure S8, the over-representation of oral cavity bacteria in the guts of PPI users is depicted in a cladogram.
PPI use is independent of bowel movement frequency and stool consistency

Some of the significantly increased taxa were more abundant in the small intestine. To ensure that the changes observed in microbiota composition were not due to diarrhoea and/or more frequent bowel movements, we checked in our general population whether clinical symptoms of diarrhoea were more often present in PPI users. Neither diarrhoeal complaints (IBS-D and functional diarrhoea, p=0.22, Fisher’s exact test), stool consistency as defined by the Bristol Stool Scale (r=0.027, p=0.36, Spearman’s correlation) nor the defecation frequency (r=−0.001, p=0.98, Spearman’s correlation) of the participants in Cohort 1 were related to PPI use.

PPI, ANTIBIOTICS AND OTHER COMMONLY USED DRUGS

In Cohort 1, 16 taxa were associated with antibiotics and other commonly used drug categories besides PPI (see online supplementary table S7). After correction for PPI use, only six taxa remained associated with certain drugs: statins, fribates and drugs that change bowel movements. All 92 alterations in bacterial taxa associated with PPI use remained statistically significant if we correct the microbiome analyses for antibiotics and other commonly used drugs.

CONCLUSIONS

We show that PPI use is consistently associated with profound changes in the gut microbiome. In our study, these changes were more prominent than changes associated with either antibiotics or other commonly used drugs. While PPIs have proven to be useful in the prevention and treatment of ulcers and GORD, they have also been associated with an increased risk of C. difficile, Salmonella spp, Shigella spp, Campylobacter spp, and other enteric infections. The increased risk of acquiring one of these enteric infections is likely due to changes in the PPI user’s gut microbiome. Gut microbiota can resist or promote colonisation of C. difficile and other enteric infections through mechanisms that either directly inhibit bacterial growth or enhance the immune system. In the case of C. difficile, spores might be able to germinate more easily because of metabolites synthesised by certain gut bacteria.

We hypothesised that PPIs change the gut microbiome through their direct effect on stomach acid. This acidity forms one of the main defenses against the bacterial influx that accompanies ingesting food and oral mucus. PPIs reduce the acidity of the stomach, allowing more bacteria to survive this barrier. We have shown here that species in the oral microbiome are more abundant in the gut microbiome of PPI users. Moreover, a study looking into the effect of PPIs on the oesophageal and gastric microbiome in oesophagitis and Barrett’s oesophagus showed similar bacterial taxa associated with PPI use, including increased levels of Enterobacteriaceae, Micrococcaceae, Actinomycetaceae and Erysipelotrichaceae. Gastric bypass surgery compromises the stomach acid barrier and leads to gut microbiome changes similar to the PPI-associated alterations in this study, thereby supporting our hypothesis.

We looked at the role of the gut microbiome in C. difficile infections, which cause 12.1% of all nosocomial infections and are responsible for half a million infections and associated with 29 000 deaths in the USA in 2011. Virulent strains of C. difficile can only colonise a susceptible gut, after which toxins are produced and spores are shed. This leads to a wide spectrum of symptoms varying from mild diarrhoea to fulminant relapsing diarrhoea and pseudomembranous colitis. Recent human, animal and in vitro studies show an overlap between the specific alterations in the gut microbiota associated with PPI use found in this study and bacterial changes that lead to increased susceptibility to C. difficile. The reduced α diversity in PPI-users is associated with increased susceptibility to C. difficile infection. The PPI-associated decreases of the family Ruminococcaceae and the genus Bifidobacterium, as well as the PPI-associated increases of the class Gammaproteobacteria, the families Enterobacteriaceae, Enterococcaceae, Lactobacillaceae and the genera Enterococcus and Veillonella, have been consistently linked to increased susceptibility to C. difficile infection.

The Ruminococcaceae family is significantly decreased in patients with C. difficile and enriched in healthy controls. Moreover, mice that have been treated with a mixture of antibiotics that do not become clinically ill after a challenge with C. difficile have higher levels of Ruminococcaceae. Within the Ruminococcaceae family, the Faecalibacterium genus was significantly increased in patients who recovered from C. difficile illness, whereas it was severely decreased in patients with C. difficile with active disease. Last, a decreased Ruminococcus torques operational taxonomic unit (OTU) was significantly associated with C. difficile infection in another study, although their OTU picking was done using a different reference database and associations were performed using OTU level, making direct comparisons with our study difficult.

Species of the Bifidobacterium genus: Bifidobacterium longum, Bifidobacterium lactis, Bifidobacterium pseudocatenulatum, Bifidobacterium breve, Bifidobacterium pseudolongum, Bifidobacterium adolescentis and Bifidobacterium animalis lactis have been shown to inhibit or prevent C. difficile infection. The administration of antibiotics that enhance the susceptibility to C. difficile in an in vitro model of the gut also significantly reduce the genus Bifidobacterium. Moreover, active C. difficile diarrhoea is associated with decreased Bifidobacteria in elderly patients.

The class Gammaproteobacteria and the family Enterobacteriaceae are significantly increased in PPI users. Gammaproteobacteria are enriched in patients with C. difficile compared with healthy controls. Within the class Gammaproteobacteria, the family Enterobacteriaceae dominate the murine gut microbiome after administration of clindamycin. Those mice that became clinically ill after the administration of antibiotics that enhance the susceptibility to C. difficile challenge, had profoundly increased levels of Enterobacteriaceae in their gut microbiome, while mice that did not become clinically ill had a gut microbiome that predominantly consisted of Firmicutes. The family Enterobacteriaceae is also increased in hamsters that were treated with clindamycin and subsequently infected with C. difficile.

The Enterococcus genus, which is also more abundant in PPI users, is significantly enriched in C. difficile-infected patients compared with healthy controls. An Enterococcus faecalis OTU and an Enterococcus avium OTU are significantly associated with increased susceptibility to C. difficile infections in mice. Moreover, an Enterococcus avium OTU is also significantly associated with C. difficile in humans. The administration of the antibiotic ceftriaxone led to an increase in the genus Enterococcus and enhanced the susceptibility to C. difficile in an in vitro model of the gut.

The increased abundance of the family Lactobacillaceae in PPI users was associated with increased risk of C. difficile infection in several studies. Mice treated with a cocktail of antibiotics (consisting of kanamycin, gentamycin, colistin, metronidazole

and vancomycin), ceftoperazone or a combination of clindamycin and ceftoperazone have higher levels of Lactobacillaceae in their gut.36 Mice treated with ceftoperazone and clindamycin that developed C. difficile infection after being challenged with the pathogen also had a higher level of Lactobacillaceae. Within the Lactobacillaceae family, the Lactobacillus genus is significantly enriched in patients with C. difficile infection compared with healthy controls.28 Lactobacillus spp in the gut microbiome are also associated with active C. difficile diarrhoea in patients.29 In contrast to these studies, the Lactobacillus species Lactobacillus delbrueckii, Lactobacillus plantarum and a Lactobacillus reuteri OTU increased colonisation resistance to C. difficile.30,31 However, in concordance with increased risk, a Lactobacillus johnsonii OTU enhanced C. difficile infection.32 Last, the Veillonella genus that is increased in PPI users is significantly enriched in patients with C. difficile compared with healthy controls.28

The prevention of healthcare-associated C. difficile infections is a priority in the USA and reduction targets for 2020 have been established.32 33 A recent study looking into the effect of PPI on the risk of developing recurrent C. difficile infections found that of 191 PPI users admitted to a hospital, only 47.1% had an evidence-based indication for PPI use.34 Moreover, PPI use was discontinued in only 0.6% of the cases.35 The US Food and Drug Administration already recommends limiting PPI use to a minimum dose and duration.36 Despite these recommendations, PPIs are still often overprescribed.37 38 The risk of unnecessary antibiotics use is already addressed.39 However, limiting the unnecessary use of PPI should also be considered in preventing C. difficile and other enteric infections.

The microbiome is being intensively studied in various diseases and conditions including IBD, IBS, obesity, old age, non-alcoholic steatohepatitis and non-alcoholic fatty liver disease (NAFLD).40 PPI users are over-represented in these groups as they more likely have to GI complaints or experience GORD, either due to their health condition or their associated lifestyle. Prominent microbiome studies looking into obesity, IBD and NAFLD include results that researchers have contributed to the condition under study, but we show they are also associated with PPI use.17 37 It could well be that some of the observed effects should rather have been attributed to the use of PPI. Future microbiome studies in humans should therefore always take the effect of PPI on the gut microbiome into account.

This paper reports the largest study to date investigating the influence of PPI on the gut microbiome. The profound alterations seen in the gut microbiome could be linked to the increased risk of C. difficile and other enteric infections. Given the widespread use of PPI, the morbidity and mortality associated with enteric infections, and the increasing number of studies investigating the microbiome, healthcare practitioners and microbiome researchers should be fully aware of the influence of PPI on the gut microbiome.

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Contributors AZ, RKW, CW and DI designed the study. FI, EFT, SAJ, ZM, LV, MCC and GD acquired the data; FI, MJB and AV analysed and interpreted the data; FI drafted the manuscript; MJB and AV performed the statistical analysis; AZ, RKW, CW, DI, GD, LF, JF, HMJRx and RJX critically reviewed the manuscript; AZ, RKW, CW, DJ, LF and JF obtained funding; AZ, RKW, CW and DJ supervised the study.

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Competing interests None declared.

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19 Caporaso JG, Kuczynski J, Stombaugh J, et al. Correspondence QIIME allows analysis of high-throughput community sequencing data intensity


Supplementary Appendix

Proton Pump Inhibitors Affect the Gut Microbiome


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Index Supplementary Appendix

- Supplementary Appendix. Online Methods
- Supplementary Table S1. Taxonomic comparison of cohort 1,2 and 3
- Supplementary Table S2. Outcome meta-analysis: All bacterial taxa
- Supplementary Table S3. Outcome meta-analysis: Annotation
- Supplementary Table S4: MaAsLin results: Cohort 1 LifeLines-DEEP
- Supplementary Table S5. MaAsLin results: Cohort 2 IBD UMCG
- Supplementary Table S6. MaAsLin results: Cohort 3 IBS MUMC
- Supplementary Table S7. Cohort 1 medication influencing the microbiome
- Supplementary Figure S1. Bar charts: Gut microbiome composition phylum level
- Supplementary Figure S2. Bar charts: Gut microbiome composition class level
- Supplementary Figure S3. Alpha diversity: Shannon index
- Supplementary Figure S4. Alpha diversity: Richness
- Supplementary Figure S5. Heatmap all significant associated taxa in all cohorts
- Supplementary Figure S6. PCoA component 1 and component 3
- Supplementary Figure S7. PCoA separate for individual cohorts.
- Supplementary Figure S8. Cladogram: Oral cavity bacteria marked
Supplementary Methods

Cohorts

Cohort 1: General Population; LifeLines-DEEP

LifeLines-DEEP is a sub-cohort of LifeLines: a multi-disciplinary prospective population-based study that examines health and health-related behaviors of 167,729 participants in the North of The Netherlands. A subset of approximately 1,500 LifeLines participants also takes part in LifeLines-DEEP. These participants are examined more thoroughly, specifically with respect to molecular data. This allows for more in-depth investigation of the genetic and phenotypic variation. Additional biological materials as well as additional information on environmental factors are collected. For a subset of 116 LifeLines participants, oral microbiome data is available as well. Consumption of PPI was obtained from questionnaire data. Analysis of gut complaints and presence of Irritable Bowel Syndrome (IBS) in LifeLines-DEEP cohort were defined according to the Rome III criteria. The 116 LifeLines-DEEP participants from which oral microbiome samples were collected, were used to compare the gut microbiome compositions to oral composition.

Cohort 2: IBD patients; Gastroenterology UMCG

The Groningen IBD Microbiome Cohort consists of more than 309 IBD patients from the Gastroenterology and Hepatology department of the University Medical Center Groningen. Stool samples are collected from these patients.
Cohort 3: IBS Case-Control

In Cohort 3, 193 IBS patients, between 18 and 75 years of age, were diagnosed by their general practitioner using the ROME III criteria. Patients were recruited via the outpatient gastroenterology clinic of the MUMC+ (Maastricht University Medical Center, a secondary and tertiary referral centre) and via general practitioners in the area of Maastricht. Medical history was taken and GI endoscopy with biopsies, abdominal imaging and/or blood, breath and fecal analyses were performed to exclude organic disease, if indicated. Patients with a history of abdominal surgery, except appendectomy, laparoscopic cholecystectomy and hysterectomy, were excluded. Patients were assigned to IBS subtypes based on predominant bowel habits according to the ROME III criteria: diarrhea (IBS-D), constipation (IBS-C), mixed stool pattern (IBS-M) and unspecified subtype (IBS-U). Age and sex matched healthy controls (HC, n=152) were enrolled via public advertising. A brief medical history was taken to exclude the presence of previous or current GI disorders or complaints. All study participants gave written informed consent prior to inclusion, and completed questionnaires regarding demographic characteristics and lifestyle factors. A 14-day end-of-day GI symptom diary was completed, addressing symptoms GI complaints and medication information.

PPI definition

The PPI users were defined as users of: Omeprazol (Lozec), Esomeprazol (Nexium), Pantoprazol (Pantozol). In cohort 1 (LifeLines-Deep) the information of drugs usage was extracted from standardized questionnaire. In cohort 2 (IBD) drug analysis was performed
based on electronic patient's record. PPI consumption in IBS case-control cohort was performed based on self-reported questionnaires.

Definition of other drug groups (% of users in cohort 1)

(1) medication that changes bowel movement or stool frequency (2.8%)
   a. Opiates (can cause severe constipation)
   b. Laxatives

(2) lowers triglycerides levels (0.2%)
   a. Fibrates

(3) lowers cholesterol levels (3.6%)
   a. Statins

(4) anti-diabetic medication (both oral and insulin) (1.2%)
   a. Insulin
   b. Biguanides
   c. Sulfonylureas
   d. α-glucosidase Inhibitors
   e. Dipeptidyl Peptidase-IV Inhibitors
   f. Glinides
   g. Thiazolidinediones

(5) systemic anti-inflammatory medication (does not include NSAIDs)(0.6%)
   a. Thiopurines
   b. Methotrexate
   c. TNF-alpha inhibitors
d. Steroids

(6) topical anti-inflammatory medication (4.1%)
   a. steroid cream, steroid nose drops or steroid nose spray

(7) systemic antibiotics, including antifungal and antimalarial medication (1.1%)

(8) antidepressants (3.2%)
   a. SSRIs
   b. SNRIs
   c. Mirtazapine
   d. Tricyclic antidepressants

**Fecal sample collection**

Fecal samples in all three cohorts were collected at home and immediately stored at -20°C. After transport on dry ice, all samples were stored at -80°C. Aliquots were made and DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen; cat. # 80204). Isolated DNA was sequenced at the Broad institute as described below.

**Oral sample collection**

Oral cavity samples were collected using sterilized cotton swabs. The cotton swabs were placed directly in tubes containing 300 µl MicroBead solution and stored at −80 ºC before DNA extraction. DNA isolation from oral swabs was performed using the UltraClean microbial DNA isolation kit (cat.# 12224) from MoBio Laboratories, Carlsbad, CA. Isolated DNA was stored at −20 ºC and sequenced at the Broad institute as described below.
Sequencing

Illumina MiSeq paired end sequencing was used to determine the bacterial composition of the fecal and oral samples. Hyper-variable region V4 was selected using forward primer 515F [GTGCCAGCMGCCGCGGTAA] and reverse primer 806R [GGACTACHVGGGTWTCTAAT]. We used custom scripts to remove the primer sequences and align the paired end reads. Details on the process can be found in Gevers et al.4.

OTU-picking

The operational taxonomic unit (OTU) formation was performed using the QIIME reference optimal picking, which uses UCLUST5 (version 1.2.22q) to perform the clustering at 97% similarity. As a reference database we used a primer-specific version of the full Greengenes 13.86. Using TaxMan7, we created the primer-specific reference database containing only reference entries that matched the selected primers. During this process we restricted probe-reference mismatches to a maximum of 25%. The 16S regions that were captured by our primers, including the primer sequences, were extracted from the full 16S sequences. For each of the unique 16S sequences present in the reference database after primer selection, we determined the shared taxonomic label of the original sequences. This overlapping part of the taxonomy was used as the taxonomic label of the sequence. This process is based on work described in Bonder et al.8 and Brandt et al.7.

Statistical analysis

QIIME9 was used for exploratory analysis on the microbiome datasets, gathering basic statistics, Shannon index, richness calculation and PCoA creation. PCoA metrics were
calculated using the Bray-Curtis dissimilarity statistic implemented in QIIME. Both alpha and beta diversity were calculated in a single rarefaction of the abundance table, setting the read depth at 10,000 reads. Differences in diversity, PCoA scores and other factors associated to PPI differences were performed using the Wilcoxon test, the Spearman correlation, Fischer exact test and the chi-squared test as implemented in R\textsuperscript{10}.

In the PCoA analysis, we compared the PPI group to the non-PPI group in terms of their scores on the first three components using a Wilcoxon test. After the test we compared the group averages to determine the direction of the shift.

To identify differentially abundant taxa between the PPI users and the non-PPI users we used MaAsLin\textsuperscript{11}. In the analysis performed in MaAsLin we did not test individual OTUs, instead we focused on the most detailed taxonomic label each OTU represented. Using QIIMETOMAASLIN\textsuperscript{12} tool we aggregated the OTUs if the taxonomic label was identical, moreover if multiple OTUs represented a higher order taxa we added this higher order taxa to the analysis. In this process we compress 60,000 OTUs to 1450 separate taxonomic units. We removed taxa from the tests if they were not present in at least 1% of our samples. During the conversion from OTU to taxonomies, QIIMETOMAASLIN transformed the microbiome data to percentages after which these percentages were arcsin square root transformed. This transforms the percentages into a normal distribution.

MaAsLin uses a boosted, additive general linear model to discriminate between groups of data. We used two different MaAsLin runs, correcting for different factors. We forced the cofactors: age, gender, BMI, read-depth and antibiotic usage and turned off the automatic filtering of input taxonomies. We did not remove any taxonomic inputs during the analysis to enable the meta-analysis.
GraPhlAn was used to represent the significant results from the MaAsLin analysis in cladograms.

**Meta-analysis**

The individual analyses were combined in a meta-analysis. For the meta-analysis a weighted Z-score approach was used. The individual P-values derived from the tests were transformed to Z-scores and the direction was taken from the coefficient of the test. The Z-scores were weighted according to the sample size and combined in a meta Z-score, this was subsequently transformed in a meta P-value.

**Multiple testing correction**

For correction for multiple testing in individual analyses and in meta-analysis we used the Q-value calculated using the R Q-value package\textsuperscript{13}. The Q-value is the minimal false discovery rate at which a test may be called significant. A Q-value of 0.05 was used as the as the cut-off point for significance.
References


Supplementary Tables

1. Supplementary Table S1: Taxonomic comparison of cohort 1,2 and 3. Comparison of the taxonomic distributions in the datasets, including PPI status.
   a. File: SupplementaryTableS1_barplots_L2-L_IBD_LLD_MIBS_PPI.xlsx

2. Supplementary Table S2: Outcome meta-analysis: All bacterial taxa
   a. File: SupplementaryTableS2_MetaAnalysis.xlsx

3. Supplementary Table S3: Outcome meta-analysis: Annotation
   a. File: SupplementaryTableS6_MetaAnalysis_Sorted_Significantonly.xlsx

4. Supplementary Table S4: MaAsLin results: Cohort 1 LifeLines DEEP
   a. File: SupplementaryTableS4_Cohort1_MaAsLin_PPI.xlsx

5. Supplementary Table S5: MaAsLin results: Cohort 2 IBD UMCG
   a. File: SupplementaryTableS5_Cohort2_MaAsLin_PPI.xlsx

6. Supplementary Table S6: MaAsLin results: Cohort 3 IBS MUMC
   a. File: SupplementaryTableS6_Cohort3_MaAsLin_PPI.xlsx

7. Supplementary Table S7: Cohort 1 medication influencing the microbiome
   a. File: SupplementaryTableS7_Cohort1_FullMedicationCheck.xlsx
Supplementary Figures

Supplementary Figure S1

Comparison of the three datasets on phylum level. Cohort 1: LifeLines-DEEP, Cohort 2: IBD UMCG, Cohort 3: IBS MUCM. PPI are the PPI users, nPP are the non-PPI users.
Supplementary Figure S2
Comparison of the three datasets on class level. Cohort 1: LifeLines-DEEP, Cohort 2: IBD UMCG, Cohort 3: IBS MUCM. PPI are the PPI users, nPP are the non-PPI users.
Supplementary Figure S3:

Shannon diversity: Gut microbiome diversity is significantly diminished in PPI users. (p = 0.01)
Supplementary Figure S4:

Richness: gut microbiome of PPI-users contains significantly less observed species. (p = 0.02)
Supplementary Figure S5:

Significantly altered taxa in PPI users is consistent in three cohorts. Meta-analysis of three independent cohorts comprising 1815 fecal samples. The heatmap shows 92 significantly increased or decreased taxa associated with PPI use in the gut microbiome for each cohort and for the meta-analysis (meta-analysis FDR < 0.05).
Supplementary Figure S6:

Principal Coordinate Analysis: The gut microbiome of PPI users (red dots) is significantly different to non-PPI users (blue dots) in the first coordinate (PCoA1: $P = 1.39 \times 10^{-20}$, Wilcoxon test) and third coordinate (PCoA3: $P = 0.0004$, Wilcoxon test).
Supplementary Figure S7:

PCoA with separate colors for the individual cohorts. The gut microbiome of PPI users was significantly closer to the oral microbiome.
Supplementary Figure S8:

Cladogram of PPI-associated changes in the gut microbiome. Red dots represent significantly increased bacterial taxa. Blue dots represent significantly decreased bacterial taxa (q < 0.05). Yellow areas represent bacterial taxa of the gut microbiome that are more abundantly present in the oral microbiome.